TITLE OF THE INVENTION

ADENOVIRUS SEROTYPE 24 VECTORS, NUCLEIC ACIDS AND VIRUS PRODUCED THEREBY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefits of U.S. provisional application serial no. 60/455,312, filed March 17, 2003

BACKGROUND OF THE INVENTION

Adenoviruses are nonenveloped, icosahedral viruses that have been identified in several avian and mammalian hosts; Horne *et al.*, 1959 *J. Mol. Biol.* 1:84-86; Horwitz, 1990 In *Virology*, eds. B.N. Fields and D.M. Knipe, pps. 1679-1721. The first human adenoviruses (Ads) were isolated over four decades ago. Since then, over 100 distinct adenoviral serotypes have been isolated which infect various mammalian species, 51 of which are of human origin; Straus, 1984, In *The Adenoviruses*, ed. H. Ginsberg, pps. 451-498, New York:Plenus Press; Hierholzer *et al.*, 1988 *J. Infect. Dis.* 158:804-813; Schnurr and Dondero, 1993, *Intervirology*;36:79-83; Jong *et al.*, 1999 *J Clin Microbiol.*, 37:3940-5. The human serotypes have been categorized into six subgenera (A-F) based on a number of biological, chemical, immunological and structural criteria which include hemagglutination properties of rat and rhesus monkey erythrocytes, DNA homology, restriction enzyme cleavage patterns, percentage G+C content and oncogenicity; Straus, *supra*; Horwitz, *supra.*

The adenovirus genome is very well characterized. It consists of a linear double-stranded DNA molecule of approximately 36,000 base pairs, and despite the existence of several distinct serotypes, there is some general conservation in the overall organization of the adenoviral genome with specific functions being similarly positioned.

Adenovirus has been a very attractive target for delivery of exogenous genes. The biology of adenoviruses is very well understood. Adenovirus has not been found to be associated with severe human pathology in immuno-competent individuals. The virus is extremely efficient in introducing its DNA into the host cell and is able to infect a wide variety of cells. Furthermore, the virus can be produced at high virus titers in large quantities. In addition, the virus can be rendered replication defective by deletion of the essential early-region 1 (El) of the viral genome; Brody et al, 1994 *Ann N Y Acad Sci.*, 716:90-101.

Replication-defective adenovirus vectors have been used extensively as gene transfer vectors for vaccine and gene therapy purposes. These vectors are propagated in cell

lines that provide E1 gene products *in trans*. Supplementation of the essential E1 gene products *in* trans is very effective when the vectors are from the same or a very similar serotype. E1-deleted group C serotypes (Ad1, Ad2, Ad5 and Ad6), for instance, grow well in 293 or PER.C6 cells which contain and express the Ad5 E1 region. However, the Ad5 E1 sequences in 293 or PER.C6 cells do not fully complement the replication of all serotypes other than group C. This is perhaps due to the inability of the Ad5 (group C) E1B 55K gene product to functionally interact with the E4 gene product(s) of the non-group C serotypes. Although the interaction is conserved within members of the same subgroup, it has not been found to be well conserved between subgroups. In order to successfully and efficiently rescue recombinant adenovirus of alternative, non-group C serotypes, a cell line expressing the E1 region of the serotype of interest would have to be generated. Alternatively, available Ad5E1-expressing cell lines could be modified to express Ad5E4 (or Orf6) in addition to Ad5E1. These additional, sometimes tedious and daunting tasks, impeded the production of recombinant, non-group C adenoviral vectors.

An efficient means for the propagation and rescue of alternative serotypes in an Ad5 E1-expressing cell line (such as PER.C6 or 293) was disclosed in pending U.S. provisional application (Serial No. 60/405,182, filed August 22, 2002). This method involves the incorporation of a critical E4 region into the adenovirus to be propagated. The critical E4 region is native to a virus of the same or highly similar serotype as that of the E1 gene product(s), particularly the E1B 55K region, of the complementing cell line, and comprises, in the least, nucleic acid encoding E4 Orf6.

Presently, two well-characterized adenovirus serotypes from subgroup C, Ad5 and Ad2, are the most widely used gene delivery vectors. There is a need to develop alternate Ad serotypes as gene transfer vectors since neutralizing antibodies in the general population may limit primary dosing or redosing with the same serotype. The prevalence of neutralizing antibody can vary from serotype to serotype. Neutralizing antibodies to some serotypes such as Ad5 are common, while antibodies to others are relatively rare. Alternate serotypes, furthermore, possess alternate tropisms which may lead to the elicitation of superior immune responses when used for vaccine or gene therapy purposes.

Adenovirus serotype 24, a subgroup D adenovirus, was originally isolated in 1960 (S.D. Bell *et al.*, 1960 *Am. J. Trop. Hyg.* 9:523) and established as a recognized reference strain in 1963 (H.G. Pereira *et al.*, 1963 *Virology* 20:613). Its antigenic relationship to 46 other human adenoviruses determined in reference horse antisera has been discussed; J.C. Hierholzer *et al.*, 1991 *Arch. Virol.* 121:179-197. The partial sequence of Ad24 hexon (1091 of 2838 bp) was disclosed in Takeuchi *et al.*, 1999 *J. Clin. Microbiol.* 37:3392-3394, and deposited in Gen Bank

(accession no. AB023553). The sequence of the virus associated RNA (VA RNA) for Ad24 and partial sequence for the pre-terminal protein and 52/55K proteins (521 bp) was disclosed in Ma & Matthews, 1993 *J. Virol.* 67:6605-6617, and Gen Bank (accession No. HAU52544).

The fields of vaccines and gene therapy would greatly benefit from additional knowledge concerning alternative adenoviral serotypes, particularly those serotypes such as Ad24 which are not well represented in the human population. Of particular interest are recombinant adenoviral vectors based on alternative adenoviral serotypes, and means of obtaining such recombinant adenoviral vectors. This need in the art is met with the disclosure of the present application related to recombinant adenoviral vectors based on adenoviral serotype 24.

SUMMARY OF THE INVENTION

The present invention relates to recombinant, replication-deficient adenovirus vectors of serotype 24, a rare adenoviral serotype, and methods for generating the recombinant adenovirus based on the alternative serotype. Additionally, means of employing the recombinant adenovirus for the delivery and expression of exogenous genes are provided. The invention, thus, encompasses recombinant, replication-defective adenoviral vectors of serotype 24 which comprise one or more transgenes operatively linked to regulatory sequences which promote effective expression of the respective transgene(s). Host administration of such recombinant adenovirus serotype 24 vectors, whether administered alone or in a combined modality and/or prime boost regimen, results in the efficient expression of the incorporated transgene and effectively induces an immune response capable of specifically recognizing the particular antigen administered (*e.g.,* HIV). Furthermore, the recombinant virus should evade pre-existing immunity to adenovirus serotypes which are more commonly encountered in the human population (*e.g.,* Ad5 and Ad2). The disclosed methods, thus, present an enhanced means for inducing an immune response against a particular antigen of interest (*e.g.,* HIV). Accordingly, the resultant immune response should offer a prophylactic advantage to previously uninfected individuals and/or provide a therapeutic effect by reducing viral load levels within an infected individual, thus prolonging the asymptomatic phase of infection.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the homologous recombination scheme utilized to recover pAd24ΔE1.

Figure 2 illustrates the homologous recombination scheme utilized to recover pAd24ΔE1Ad5Orf6.

Figure 3 illustrates the configuration of E4 regions in the Ad24 recombinants generated.

Figure 4 illustrates the growth kinetics of the Ad24-based vectors in PER.C6 cells.

Figures 5A-1 through 5A-10 illustrate the nucleic acid sequence for wild-type adenovirus serotype 24 (SEQ ID NO: 1). The ATCC product number for Ad24 is VR-259.

Figure 6 illustrates, in tabular format, gag-specific T cell responses in monkeys immunized with MRKAd5-HIVgag and Ad24 HIV vectors. Shown are the numbers of spot-forming cells per million PBMC following incubation in the absence (mock) or presence of Gag peptide pool. The pool consisted of 20-aa peptide overlapping by 10 aa and encompassing the entire gag sequence.

Figure 7 illustrates, in tabular format, the characterization of the gag-specific T cells in monkeys immunized with 10^11 vp of MRKAd5-HIV1gag and Ad24ΔE1gagΔOrf6Ad5Orf6. Shown are the percentages of CD3+ T cells that are either gag-specific CD4+ or gag-specific CD8+ cells. These values were corrected for mock values (<0.03%).

Figure 8 illustrates individual anti-p24 titers (in mMU/mL) in macaques immunized with gag-expressing adenovirus vectors.

Figure 9 illustrates *in vivo* expression of SEAP in C3H/HeN mice using 10^10 vp doses of Ad24 vectors. The vectors were injected intramuscularly and the levels of SEAP expression were determined from the serum samples. Two extra cohorts received 10^10 vp and 10^9 vp of Ad5 vector. Shown are geometric means for each cohort of 5 mice.

Figure 10 illustrates *in vivo* SEAP expression using MRKAd5 and Ad24 vectors in rhesus macaques. Shown are the geometric means of the SEAP levels for cohorts of 3 monkeys. In bars are the standard errors of the geometric means.

Figure 11 illustrates a homologous recombination scheme to be utilized to recover pAd24ΔE1ΔE4Ad5Orf6.

Figure 12 illustrates the nucleic acid sequence (SEQ ID NO: 3) of the optimized human HIV-1 gag open reading frame.

Figure 13 illustrates the nucleic acid sequence encoding the gag expression cassette (SEQ ID NO: 4). The various regions of the figure are as follows: (1) a first underlined segment of nucleic acid sequence encoding the immediate early gene promoter region from human cytomegalovirus; (2) a first segment of lowercase letters which is not underlined, which segment of DNA contains a convenient restriction enzyme site; (3) a region in caps which contains the coding sequence of HIV-1 gag; (4) a second segment of lowercase letters which is not underlined, which segment of DNA contains a convenient restriction enzyme site; and (5) a second underlined segment, this segment containing nucleic acid sequence encoding a bovine growth hormone polyadenylation signal sequence.

Figure 14 illustrates the nucleic acid sequence encoding the SEAP expression cassette (SEQ ID NO: 5). The various regions of the figure are as follows: (1) a first underlined segment of nucleic acid sequence encoding the immediate early gene promoter region from human cytomegalovirus; (2) a first segment of lowercase letters which is not underlined, which segment of DNA contains a convenient restriction enzyme site; (3) a region in caps which contains the coding sequence of the human placental SEAP gene; (4) a second segment of lowercase letters which is not underlined, which segment of DNA contains a convenient restriction enzyme site; and (5) a second underlined segment, this segment containing nucleic acid sequence encoding a bovine growth hormone polyadenylation signal sequence.

Figures 15A-1 through 15A-10 illustrate the nucleic acid sequence for wild-type adenovirus serotype 17 (SEQ ID NO: 6; Accession No. AF108105).

Figures 16A-1 through 16A-47 illustrate the nucleotide sequence of the pMRKAd5HIV-1gag vector (SEQ ID NO:7 [coding] and SEQ ID NO:8 [non-coding]).

Figures 17A-1 through 17-A-14 illustrate the nucleic acid sequence for the Ad6 genome (SEQ ID NO: 9).

Figure 18 illustrates gag-specific T cell responses in rhesus macaques immunized following a heterologous Ad5/Ad6 prime-Ad24 boost regimen. a: Mock, no peptide: gag, 20-mer peptide pool encompassing entire gag sequence; b: Peak response after 2 or 3 doses of the priming vaccine; c: 3 wks prior to boost; d: 4 wks after boost; e: ND, not determined.

Figure 19 illustrates, in tabular format, the percentages of $CD3^+$ T lymphocytes that are gag-specific $CD8^+$ cells or gag-specific $CD4^+$ cells determined after the Ad24 Boost Immunization (wk 60). Numbers reflect the percentages of circulating CD3+ lymphocytes that are either gag-specific CD4+ or gag-specific CD8+ cells. Mock values (equal to or less than 0.01%) have been subtracted.

Figure 20 illustrates gag-specific T cell responses in rhesus macaques immunized following a heterologous Ad 24 prime-Ad5 boost regimen. a: Mock, no peptide: gag, 20-mer peptide pool encompassing entire gag sequence; b: Peak response after 2 doses of the priming vaccine; c: Wk 24; d: 4 wks after boost; e: ND, not determined.

DETAILED DESCRIPTION OF THE INVENTION

Rare adenoviral serotypes possess an inherent advantage over the more commonly exploited adenoviral serotypes (for instance, adenoviral serotypes 2 and 5) since preexisting immunity is unlikely to limit their efficient delivery and expression of exogenous genes to their target site. Different adenoviral serotypes also exhibit distinct tropisms by reason of their varying capsid structure and, thus, present the potential for targeting different tissues and possibly leading to the elicitation of superior immune responses when used for vaccine or gene therapy purposes. These rare adenoviral serotypes when rendered replication-defective, however, can be difficult to propagate and rescue in currently available adenoviral propagation cell lines.

Applicants have recently managed to successfully rescue and propagate one such rare, replication-defective alternative serotype, adenovirus serotype 24, a subgroup D adenovirus, and herein demonstrate the effective functioning of the adenovirus in the delivery and expression of exogenous transgenes.

Accordingly, the present invention relates to a recombinant adenoviral vector of serotype 24 suitable for use in gene therapy or vaccination protocols. The nucleic acid sequence for wild-type adenovirus serotype 24 (SEQ ID NO: 1) is illustrated in Figures 5A-5J, although any functional homologue or different strain of adenovirus serotype 24 can be utilized in accordance with the methods of the present invention, as one of ordinary skill in the art will appreciate. Adenovirus serotypes have been distinguished through a number of art-appreciated biological, chemical, immunological and structural criteria which include hemagglutination properties of rat and rhesus monkey erythrocytes, DNA homology, restriction enzyme cleavage patterns, percentage G+C content and oncogenicity; Straus, *supra*; Horwitz, *supra*. A given serotype can be identified by a number of methods including restriction mapping of viral DNA; analyzing the mobility of viral DNA; analyzing the mobility of virion polypeptides on SDS-polyacrylamide gels following electrophoresis; comparison of squence information to known sequence particularly from capsid genes (*e.g.,* hexon) which contain sequences that define a serotype; and comparing a sequence with reference sera for a particular serotype available from the ATCC. Classification of adenovirus serotypes by SDS-PAGE has been discussed in Wadell

et al., 1980 *Ann. N.Y. Acad. Sci.* 354:16-42. Classification of adenovirus serotypes by restriction mapping has been discussed in Wadell *et al.*, *Current Topics in Microbiology and Immunology* 110:191-220. Reference sera for Ad24 are available from the ATCC (ATCC Product Nos. VR-1102AS/RB and VR-1102PI/RB). Adenovirus serotype 24, a subgroup D adenovirus, was originally isolated in 1960 (S.D. Bell *et al.*, 1960 *Am. J. Trop. Hyg.* 9:523) and has been established as a recognized reference strain in 1963 (H.G. Pereira *et al.*, 1963 *Virology* 20:613). Its antigenic relationship to 46 other human adenoviruses determined in reference horse antisera has been discussed in the art; J.C. Hierholzer *et al.*, 1991 *Arch. Virol.* 121:179-197.

Adenovirus serotype 24 vectors in accordance with the present invention are at least partially deleted in E1 and devoid (or essentially devoid) of E1 activity, rendering the vector incapable of replication in the intended host. Preferably, the E1 region is completely deleted or inactivated. The adenoviruses may contain additional deletions in E3, and other early regions, albeit in situations where E2 and/or E4 is deleted, E2 and/or E4 complementing cell lines may be required to generate recombinant, replication-defective adenoviral vectors.

Adenoviral vectors of use in the methods of the present invention can be constructed using well known techniques, such as those reviewed in Hitt *et al.*, 1997 "Human Adenovirus Vectors for Gene Transfer into Mammalian Cells" *Advances in Pharmacology* 40:137-206, which is hereby incorporated by reference. Often, a plasmid or shuttle vector containing the heterologous nucleic acid of interest is generated which comprises sequence homologous to the specific adenovirus of interest. The shuttle vector and viral DNA or second plasmid containing the cloned viral DNA are then co-transfected into a host cell where homologous recombination occurs and results in the incorporation of the heterologous nucleic acid into the viral nucleic acid. Preferred shuttle vectors and cloned viral genomes contain adenoviral and plasmid portions. For shuttle vectors used in the construction of replication-defective vectors, the adenoviral portion typically contains non-functional or deleted E1 and E3 regions and the gene expression cassette, flanked by convenient restriction sites. The plasmid portion of the shuttle vector typically contains an antibiotic resistance marker under the transcriptional control of a prokaryotic promoter. Ampicillin resistance genes, neomycin resistance genes and other pharmaceutically acceptable antibiotic resistance markers may be used. To aid in the high level production of the nucleic acid by fermentation in prokaryotic organisms, it is advantageous for the shuttle vector to contain a prokaryotic origin of replication and be of high copy number. A number of commercially available prokaryotic cloning vectors provide these benefits. Non-essential DNA sequences are, preferably removed. It is also preferable that the vectors not be able to replicate in eukaryotic cells. This minimizes the risk of

integration of nucleic acid vaccine sequences into the recipients' genome. Tissue-specific promoters or enhancers may be used whenever it is desirable to limit expression of the nucleic acid to a particular tissue type.

Homologous recombination of the shuttle vector and wild-type adenovirus 24 viral DNA (Ad24 backbone vector) results in the generation of adenoviral pre-plasmids (see, for instance, pAd24ΔE1, pAd24ΔE1Ad5Orf6, pAd24ΔE1gagΔE4Ad5Orf6, pAd24ΔE1gagΔOrf6Ad5Orf6, pAd24ΔE1SEAPΔE4Ad5Orf6, pAd24ΔE1SEAPΔOrf6Ad5Orf6). Upon linearization, the pre-plasmids are capable of replication in PER.C6® cells or alternative E1-complementing cell lines. Infected cells and media can then be harvested once viral replication is complete.

A packaging cell will generally be needed in order to produce sufficient amount of adenovirus. The packaging cell should contain elements which are necessary for the production of the specific adenovirus of interest. It is preferable that the packaging cell and the vector not contain overlapping elements which could lead to replication competent virus by recombination. Specific examples of cells which are suitable for the propagation of recombinant Ad24 E1-deleted vectors express the early region 1 (E1) of adenovirus 24 or another group D serotype. Alternatively, propagation cell lines can be used which express adenoviral E1 and E4 regions (particularly, E4 open reading frame 6 ("ORF6")) which are derived from the same serotype but different subgroup than Ad24 (*e.g.,* Ad5 E1 and E4); *see, e.g.,* Abrahamsen *et al.,* 1997 *J. Virol.* 8946-8951, and U.S. Patent No. 5,849,561. Additionally, a cell line could be used that expresses E1B from Ad24 in addition to (1) E1A or (2) E1A and E1B from a serotype of a different subgroup. In copending U.S. provisional application serial no. 60/405,182, filed August 22, 2002, a strategy was disclosed for the efficient propagation and rescue of alternative adenoviral serotypes. The method is based on incorporating, into the genome of the adenovirus vector, an E4 region (or portion thereof including E4 ORF6) of the same or highly similar serotype as that of the E1 gene product(s), particularly E1B, being expressed by the complementing cell line. Examples 1-4 demonstrate the viability of such a method through the incorporation of an Ad5E4 region and its propagation in PER.C6 cells (which cells express Ad5E1). The wildtype adenovirus serotype 5 sequence is known and described in the art; *see* Chroboczek *et al.*, 1992 *J. Virol.* 186:280, which is hereby incorporated by reference. Placement of the E4 region or ORF6-containing portion is not critical; *see* Examples 1-4. The critical step is making sure that either a promoter is supplied or the gene is strategically placed so that it runs off a promoter native to the vector (*e.g.,* such as the E4 promoter). The native E4 region of the

vector can be replaced, deleted or left intact. This method is, thus, suitable for use in the propagation and rescue of the adenoviral vectors of the present invention.

Typically, propagation cells are human cells derived from the retina or kidney, although any cell line capable of expressing the appropriate E1 and/or E4 region(s) can be utilized in the present invention. Embryonal cells such as amniocytes have been shown to be particularly suited for the generation of El complementing cell lines. Several cell lines are available. These include but are not limited to the known cell lines PER.C6 (ECACC deposit number 96022940), 911, 293, and El A549.

The present invention encompasses methods for producing a recombinant, replication-defective adenovirus of serotype 24 in an adenoviral E1-complementing cell line, comprising transfecting a recombinant, replication-defective adenoviral vector of serotype 24 in an adenoviral E1-complementing cell and allowing for the production of viral particles. The viral particles so produced form another aspect of the present invention. Host cells comprising the recombinant, replication-defective adenoviral serotype 24 vectors of the present invention form yet another aspect of the present invention; host cells being defined as a population of cells not including a transgenic human being. Recombinant, replication-defective adenovirus harvested in accordance with the methods of the present invention are encompassed herein as well. This harvested material may be purified, formulated and stored prior to host administration.

Adenoviral vectors in accordance with the present invention are very well suited to effectuate expression of desired proteins, especially in situations where an individual's immune response effectively prevents administration or readministration via the more commonly employed adenoviral serotypes. Accordingly, specific embodiments of the present invention are recombinant, replication-defective adenoviral vectors of serotype 24 which comprise a heterologous nucleic acid of interest. The nucleic acid of interest can be a gene, or a functional part of a gene. The nucleic acid can be DNA and/or RNA, can be double or single stranded, and can exist in the form of an expression cassette. The nucleic acid can be inserted in an E1 parallel (transcribed 5' to 3') or anti-parallel (transcribed in a 3' to 5' direction relative to the vector backbone) orientation. The nucleic acid can be codon-optimized for expression in the desired host (*e.g.*, a mammalian host). The heterologous nucleic acid can be in the form of an expression cassette. A gene expression cassette will typically contain (a) nucleic acid encoding a protein or antigen of interest; (b) a heterologous promoter operatively linked to the nucleic acid encoding the protein; and (c) a transcription termination signal.

In specific embodiments, the heterologous promoter is recognized by an eukaryotic RNA polymerase. One example of a promoter suitable for use in the present invention is the immediate early human cytomegalovirus promoter (Chapman *et al.*, 1991 *Nucl. Acids Res.* 19:3979-3986). Further examples of promoters that can be used in the present invention are the strong immunoglobulin promoter, the EF1 alpha promoter, the murine CMV promoter, the Rous Sarcoma Virus promoter, the SV40 early/late promoters and the beta actin promoter, albeit those of skill in the art can appreciate that any promoter capable of effecting expression in the intended host can be used in accordance with the methods of the present invention. The promoter may comprise a regulatable sequence such as the Tet operator sequence. Sequences such as these that offer the potential for regulation of transcription and expression are useful in instances where repression of gene transcription is sought. The adenoviral gene expression cassette may comprise a transcription termination sequence; specific embodiments of which are the bovine growth hormone termination/polyadenylation signal (bGHpA) or the short synthetic polyA signal (SPA) of 50 nucleotides in length defined as follows: AATAAAAGATCTTTATTTTCATTAGATCTGTGTGTT-GGTTTTTTGTGTG (SEQ ID NO:2). A leader or signal peptide may also be incorporated into the transgene. In specific embodiments, the leader is derived from the tissue-specific plasminogen activator protein, tPA.

Heterologous nucleic acids of interest are genes (or their functional counterparts) which encode immunogenic and/or therapeutic proteins. Preferred therapeutic proteins are those which elicit some measurable therapeutic benefit in the individual host upon administration. Preferred immunogenic proteins are any proteins which are capable of eliciting an immune response in an individual. Applicants have exemplified the delivery of a representative immunogenic protein (HIV gag) in the present specification in non-human primates (rhesus macaques), albeit any gene encoding a therapeutic or immunogenic protein can be used in accordance with the methods disclosed herein. The adenovirus serotype 24 vectors were found to induce significant levels of gag-specific T cells; Figure 6. Moreover, the results indicated that immunization with the disclosed vectors was able to elicit both HIV-specific CD4+ and CD8+ T cells; Figure 7. Additionally, detectable levels of circulating anti-gag antibodies were generated in response to administration of the vector at a dose of 10^11 vp; Figure 8.

An aspect of the present invention, therefore, relates to adenovirus serotype 24-based vectors carrying an HIV transgene. In these embodiments, nucleic acid encoding any HIV antigen may be utilized (specific examples of which include gag, pol, nef, gp160, gp41, gp120, tat, and rev, including derivatives of the aforementioned genes). The embodiments exemplified herein employ nucleic acid encoding a codon-optimized p55 gag antigen; *see* Figure 12 (SEQ ID

NO: 3). Codon-optimized HIV-1 env genes are disclosed in PCT International Applications PCT/US97/02294 and PCT/US97/10517, published August 28, 1997 (WO 97/31115) and December 24, 1997, respectively. Codon-optimized HIV-1 pol genes are disclosed in U.S. Application Serial No. 09/745,221, filed December 21, 2000 and PCT International Application PCT/US00/34724, also filed December 21, 2000. Codon-optimized HIV-1 nef genes are disclosed in U.S. Application Serial No. 09/738,782, filed December 15, 2000 and PCT International Application PCT/US00/34162, also filed December 15, 2000.

In this specific embodiment of a recombinant, replication-defective Ad24 vector comprising an HIV-1 gene, the gene may be derived from HIV-1 strain CAM-1; Myers et al, eds. "Human Retroviruses and AIDS: 1995, IIA3-IIA19, which is hereby incorporated by reference. This gene closely resembles the consensus amino acid sequence for the clade B (North American/European) sequence. HIV gene sequence(s) may be based on various clades of HIV-1; specific examples of which are Clades B and C. Sequences for genes of many HIV strains are publicly available from GenBank and primary, field isolates of HIV are available from the National Institute of Allergy and Infectious Diseases (NIAID) which has contracted with Quality Biological (Gaithersburg, MD) to make these strains available. Strains are also available from the World Health Organization (WHO), Geneva Switzerland. It is well within the purview of the skilled artisan to choose an appropriate nucleotide sequence which encodes a specific HIV antigen, or immunologically relevant portion or modification thereof. "Immunologically relevant" as defined herein means (1) with regard to a viral antigen, that the protein is capable, upon administration, of eliciting a measurable immune response within an individual sufficient to retard the propagation and/or spread of the virus and/or to reduce the viral load present within the individual; or (2) with regards to a nucleotide sequence, that the sequence is capable of encoding for a protein capable of the above.

The present invention encompasses methods for (1) effectuating a therapeutic response in an individual and (2) generating an immune response (including a cellular-mediated immune response) comprising administering to an individual a recombinant adenovirus serotype 24 vector in accordance with the present invention. One aspect of the present invention are methods for generating an enhanced immune response against one or more antigens (bacterial, viral (*e.g.*, HIV), or other (*e.g.*, cancer)) which comprise the administration of a recombinant adenovirus serotype 24 vehicle expressing the antigen of interest. Administration of recombinant Ad24 vectors in this manner provides for improved cellular-mediated immune responses, particularly where there is pre-existing immunity in a given host to the more well-represented adenovirus serotypes (*e.g.*, Ad2 and Ad5). An effect of the improved vaccine

administration methods should be a lower transmission rate to (or occurrence rate in) previously uninfected individuals (*i.e.,* prophylactic applications) and/or a reduction in the levels of virus/bacteria/foreign agent within an infected individual (*i.e.,* therapeutic applications). As relates to HIV indications, an effect of the improved vaccine administration methods should be a lower transmission rate to previously uninfected individuals (*i.e.,* prophylactic applications) and/or a reduction in the levels of viral loads within an infected individual (*i.e.,* therapeutic applications) so as to prolong the asymptomatic phase of HIV infection. Administration, intracellular delivery and expression of the recombinant Ad24 vectors elicits a host CTL and Th response.

Accordingly, the present invention relates to methodology regarding administration of the recombinant Ad24 viral vectors (or immunogenic compositions thereof, herein termed vaccines) to provide effective immunoprophylaxis, to prevent establishment of an infection following exposure to the viral (for instance, HIV), bacterial or other agent, or as a post –infection therapeutic vaccine to mitigate infection to result in the establishment of a lower virus/bacteria/other load with beneficial long term consequences.

The recombinant adenovirus serotype 24 vectors of the present invention may be administered alone, or as part of a prime/boost administration regimen. A priming dose(s) of at least one antigen (*e.g.,* an HIV antigen) is first delivered with a recombinant adenoviral vector. This dose effectively primes the immune response so that, upon subsequent identification of the antigen(s) in the circulating immune system, the immune response is capable of immediately recognizing and responding to the antigen(s) within the host. The priming dose(s) is then followed with a boosting dose comprising a recombinant adenoviral vector containing at least one gene encoding the antigen. A mixed modality prime and boost inoculation scheme will result in an enhanced immune response, particularly where there is pre-existing anti-vector immunity. Prime-boost administrations typically involve priming the subject (by viral vector, plasmid, protein, *etc.*) at least one time, allowing a predetermined length of time to pass, and then boosting (by viral vector, plasmid, protein, *etc.*). Multiple primings, typically 1-4, are usually employed, although more may be used. The length of time between priming and boost may typically vary from about four months to a year, albeit other time frames may be used as one of ordinary skill in the art will appreciate.

In addition to a single protein or antigen of interest being delivered by the recombinant, replication-defective adenovirus serotype 24 vectors of the present invention, two or more proteins or antigens can be delivered either via separate vehicles or delivered *via* the same vehicle. Multiple genes/functional equivalents may be ligated into a proper shuttle plasmid

for generation of a pre-adenoviral plasmid comprising multiple open reading frames. Open reading frames for the multiple genes/functional equivalents can be operatively linked to distinct promoters and transcription termination sequences. In other embodiments, the open reading frames may be operatively linked to a single promoter, with the open reading frames operatively linked by an internal ribosome entry sequence (IRES; as disclosed in WO 95/24485), or suitable alternative allowing for transcription of the multiple open reading frames to run off of a single promoter. In certain embodiments, the open reading frames may be fused together by stepwise PCR or suitable alternative methodology for fusing together two open reading frames. Due consideration must be given, however, to the effective packaging limitations of the viral vehicle. Adenovirus, for instance, has been shown to exhibit an upper cloning capacity limit of approximately 105% of the wildtype Ad5 sequence.

Prime-boost regimens can employ different adenoviral serotypes. One example of such a protocol would be a priming dose(s) comprising a recombinant adenoviral vector of a first serotype followed by a boosting dose comprising a recombinant adenoviral vector of a second and different serotype; *see*, for instance, Example 11 and Figures 18, 19 and 20. Therein, a cohort of 4 macaques was given three doses of either Ad5- or Ad6-based HIV gag carrying vectors at weeks 0, 4, and 26. At week 56, a booster shot an Ad24-based vector in accordance with the present invention carrying HIV gag was delivered. Administration of the Ad24-based vector resulted in about a 13- to 47-fold enhancement in T cell responses when compared to the levels at the time of booster. In an alternative embodiment, the priming dose can comprise a mixture of separate adenoviral vehicles each comprising a gene encoding for a different protein/antigen. In such a case, the boosting dose would also comprise a mixture of vectors each comprising a gene encoding for a separate protein/antigen, provided that the boosting dose(s) administers recombinant viral vectors comprising genetic material encoding for the same or similar set of antigens that were delivered in the priming dose(s). These multiple gene/vector administration modalities can further be combined. It is further within the scope of the present invention to embark on combined modality regimes which include multiple but distinct components from a specific antigen.

Compositions, including vaccine compositions, comprising the adenoviral vectors of the present invention are an important aspect of the present invention. These compositions can be administered to mammalian hosts, preferably human hosts, in either a prophylactic or therapeutic setting. Potential hosts/vaccinees include but are not limited to primates and especially humans and non-human primates, and include any non-human mammal of commercial or domestic veterinary importance. Compositions comprising recombinant adenoviral serotype

24 vectors may be administered alone or in combination with other viral- or non-viral-based DNA/protein vaccines. They also may be administered as part of a broader treatment regimen. The present invention encompasses those situations as well where the disclosed recombinant adenoviral serotype 24 vectors are administered in conjunction with other therapies; for example, HAART therapy (in the case of a recombinant HIV vector).

Compositions comprising the recombinant viral vectors may contain physiologically acceptable components, such as buffer, normal saline or phosphate buffered saline, sucrose, other salts and polysorbate. In certain embodiments, the formulation has: 2.5-10 mM TRIS buffer, preferably about 5 mM TRIS buffer; 25-100 mM NaCl, preferably about 75 mM NaCl; 2.5-10% sucrose, preferably about 5% sucrose; 0.01 -2 mM $MgCl_2$; and 0.001%-0.01% polysorbate 80 (plant derived). The pH should range from about 7.0-9.0, preferably about 8.0. One skilled in the art will appreciate that other conventional vaccine excipients may also be used in the formulation. In specific embodiments, the formulation contains 5mM TRIS, 75 mM NaCl, 5% sucrose, 1mM $MgCl_2$, 0.005% polysorbate 80 at pH 8.0. This has a pH and divalent cation composition which is near the optimum for Ad5 and Ad6 stability and minimizes the potential for adsorption of virus to a glass surface. It does not cause tissue irritation upon intramuscular injection. It is preferably frozen until use.

The amount of viral particles in the vaccine composition to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of $1x10^7$ to $1x10^{12}$ particles and preferably about $1x10^{10}$ to $1x10^{11}$ particles is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration of interleukin-12 protein, concurrently with or subsequent to parenteral introduction of the vaccine compositions of this invention is also advantageous.

The following non-limiting Examples are presented to better illustrate the workings of the invention.

Example 1

Construction and Rescue of pAd24ΔE1.

An E1- Ad24-based pre-adenovirus plasmid was constructed in order to determine whether an E1- Ad24 vector (a representative group D serotype) could be propagated in an Ad5/group C E1-complementing cell line. Since at the time the vector construction was initiated the complete sequence of Ad24 was unknown we took advantage of some sequence homology between Ad24 and Ad17. The general strategy used to recover Ad24 as a bacterial plasmid is illustrated in Figure 1 and described below. Cotransformation of BJ5183 bacteria with purified wild-type Ad24 viral DNA and a second DNA fragment termed the Ad17 ITR cassette resulted in the circularization of the viral genome by homologous recombination. The ITR cassette contains sequences from the right (bp 34469 to 35098) and left (bp 4 to 414 and bp 3373 to 4580) end of the Ad17 genome (*see* Figures 15A-15J) separated by plasmid sequences containing a bacterial origin of replication and an Ampicillin resistance gene. The ITR cassette contains a deletion of E1 sequences from Ad17 (bp 415 to 3372) with a unique *Swa* I site located in the deletion. The Ad17 sequences in the ITR cassette provide regions of homology with the purified Ad24 viral DNA in which recombination can occur. The ITR cassette was also designed to contain unique restriction enzyme sites (*Pme* I) located at the end of the viral ITR's so that digestion will release the Ad24 genome from plasmid sequences. Potential clones were screened by restriction analysis and one clone was selected as pAd24ΔE1. pAd24ΔE1 contains Ad17 sequences from bp 4 to 414 and from bp 3373 to 4580, Ad24 bp 4588 to 34529, and Ad17 bp 34469 to 35098 (bp numbers refer to the wt sequence for both Ad17 and Ad24). PAd24ΔE1 contains the coding sequences for all Ad24 virion structural proteins that constitute its serotype specificity. This approach can be used to circularize any group D serotype into plasmid form which has sufficient homology to Ad17.

To determine if pre-adenovirus plasmid pAd24ΔE1 could be rescued into virus and propagated in a group C E1 complementing cell line, the plasmid was digested with *Pme* I and transfected into a 6 cm dish of 293 cells using the calcium phosphate co-precipitation technique. *Pme* I digestion releases the viral genome from the plasmid sequences allowing viral replication to occur after entry into 293 cells. Viral cytopathic effect (CPE), indicating that virus replication and amplification is occurring, was very slow to arise. Following multiple attempts, we were successful at rescuing and amplifying Ad24ΔE1 but the virus grew to lower titers and took more passages to amplify than a similar Ad5 based vector. In order to verify the genetic structure of the virus, viral DNA was extracted using pronase treatment followed by phenol chloroform extraction and ethanol precipitation. Viral DNA was then digested with *Hin*dIII and

treated with Klenow fragment to end-label the restriction fragments with P33-dATP. The end-labeled restriction fragments were then size-fractionated by gel electrophoresis and visualized by autoradiography. The digestion products were compared with the digestion products from the pre-plasmid (that had been digested with *Pme*1/*Hind*III prior to labeling). The expected sizes were observed, indicating that the virus had been successfully rescued.

Example 2

Insertion of Ad5 Orf 6 into the E1 region of Ad24

In order to determine if the insertion of Ad5 E4 Orf6 into the Ad24 genome would allow more efficient propagation in a group C E1 complementing cell line we constructed an Ad24 based pre-adenovirus plasmid containing Ad5 Orf6 in the E1 region. In order to introduce Ad5 Orf6 in to the E1 region of pAd24ΔE1, bacterial recombination was used. An Ad5 Orf6 transgene consisting of the Ad5 Orf6 coding region flanked by the HCMV promoter and pA was cloned into the E1 deletion in an Ad17 shuttle vector (a precursor to the Ad17 ITR cassette). The Ad5 Orf6 transgene was cloned between bp 414 and 3373 in the E1 anti-parallel orientation. The shuttle vector containing the Ad5 Orf6 transgene was digested to generate a DNA fragment consisting of the transgene flanked by Ad17 sequences (bp 4 to 414 and bp 3373 to 4580) and the fragment was purified after electrophoresis on an agarose gel. Cotransformation of BJ 5183 bacteria with the shuttle vector fragment and pAd24ΔE1, which had been linearized in the E1 region by digestion with *Swa*I, resulted in the generation of pAd24ΔE1Ad5Orf6 by homologous recombination (Figure 2). Potential clones were screened by restriction analysis and one clone was selected as pre-adenovirus plasmid pAd24ΔE1Ad5Orf6.

In order to determine if pre-adenovirus plasmid pAd24ΔE1Ad5Orf6 could be rescued into virus and propagated in an Ad5/group C E1 complementing cell line, pAd24ΔE1Ad5Orf6 was digested with *Pme* I and transfected into a 6 cm dish of 293 cells using the calcium phosphate co-precipitation technique. *Pme*I digestion releases the viral genome from plasmid sequences allowing viral replication to occur after entry into 293 cells. Once complete viral cytopathic effect (CPE) was observed at approximately 7-10 days post transfection, the infected cells and media were freeze/thawed three times and the cell debris pelleted. The virus was amplified in two additional passages in 293 cells and then purified from the final infection by ultracentrifugation on CsCl density gradients. In order to verify the genetic structure of the virus, viral DNA was extracted using pronase treatment followed by phenol

chloroform extraction and ethanol precipitation. Viral DNA was then digested with *Hin*dIII and treated with Klenow fragment to end-label the restriction fragments with P33-dATP. The end-labeled restriction fragments were then size-fractionated by gel electrophoresis and visualized by autoradiography. The digestion products were compared with the digestion products from the pre-plasmid (that had been digested with *Pme*1/*Hind*III prior to labeling). The expected sizes were observed, indicating that the virus had been successfully rescued.

Example 3

Insertion of Ad5 Orf 6 into the E4 region of Ad24

To refine the strategy of including Ad5 Orf6 in the genome of an alternative serotype so that propagation could take place in an Ad5/group C complementing cell line two additional strategies were developed. In the first strategy, the entire alternative serotype E4 region (not including the E4 promoter) was deleted and replaced with Ad5 Orf6. In the second strategy, just the alternative serotype Orf6 gene was deleted and replaced with Ad5 Orf6. The configuration of the E4 regions generated by the two strategies is diagramed in Figure 3. For each of these strategies the desired pre-Adenovirus plasmid was generated by bacterial recombination. Cotransformation of BJ 5183 bacteria with pAd24ΔOrf6BstZ17I and the appropriately constructed Ad24 E4 shuttle plasmid resulted in the generation of the desired Ad24 based pre-Ad plasmid. PAd24ΔOrf6BstZ17I, a derivative of pAd24ΔE1, was constructed so that the E4 region in the Ad24 pre-Ad plasmid could be easily modified using bacterial recombination. PAd24ΔOrf6BstZ17I contains a deletion in the E4 region from Ad24 bp 32373 to bp 33328 with a unique *Bst*Z17I site located at the position of the deletion. The complete sequence of pAd24ΔOrf6BstZ17I consists of Ad17 sequences from bp 4 to 414 and from bp 3373 to 4580, Ad24 bp 4588 to 32372 and from 33329 to 34529, and Ad17 bp 34469 to 35098 (bp numbers refer to the wt sequence for both Ad17 and Ad24).

To construct pAd24ΔE1ΔE4Ad5Orf6 (An Ad24 pre-Ad plasmid containing an E1 deletion and a deletion of E4 substituted with Ad5 Orf6), an Ad24 E4 shuttle plasmid was constructed by digesting pAd24ΔE1 with *Pme*I and *Bsr*GI and cloning the restriction fragment representing the E4 region (bp 31559 to bp 35164) into pNEB193, generating pNEBAd24E4. PNEBAd24E4 was then digested with *Acc*I and *Eco*NI to remove the E4 coding sequences and ligated with an oligo designed to contain *Bgl*II and *Xho*I sites (underlined) (5' ACTCGAGATGTATAGATCT (SEQ ID NO: 10); 5' CTAGATCTATACATCTCGAG (SEQ ID NO: 11)), generating pNEBAd24ΔE4. PNEBAd24ΔE4 was then digested with *Bgl*II and *Xho*I and ligated with the Ad5 Orf6 gene, which was PCR amplified, generating

pNEBAd24ΔE4Ad5Orf6. The PCR primers used to amplify the Ad5 Orf6 gene (5' GCACAGATCTTTGCTTCAGGAATATG (SEQ ID NO: 12); 5' GAGAACTCGAGGCCTACATGGGGGTAGAG (SEQ ID NO: 13)) were designed to contain *Bgl*II and *Xho*I sites (underlined above) for ligation with the pNEBAd24DE4 fragment. In the final step pNEBAd24ΔE4Ad5Orf6 E4 shuttle plasmid was digested with *Pvu*I and *Pme*I, the restriction fragments were size fractionated by agarose gel electrophoresis and the desired fragment containing Ad5Orf6 flanked by Ad24 sequences was gel purified. Cotransformation of BJ 5183 bacteria with E4 shuttle fragment and pAd24ΔOrf6BstZ17I, which had been linearized in the E4 region by digestion with *Bst*Z17I, resulted in the generation of pAd24ΔE1ΔE4Ad5Orf6 by homologous recombination. Potential clones were screened by restriction analysis and one clone was selected as pre-adenovirus plasmid pAd24ΔE1ΔE4Ad5Orf6.

To construct pAd24ΔE1ΔOrf6Ad5Orf6 (An Ad24 pre-Ad plasmid containing an E1 deletion and a deletion of E4 Orf6 substituted with Ad5 Orf6), an Ad24 E4 shuttle plasmid was constructed in which the Ad24 Orf6 gene was replaced by Ad5 Orf6. To do this the *Eco*R1 restriction fragment representing bp 32126 to bp 33442 of the Ad24 genome (encompassing the E4 Orf6 coding region), was subcloned into the *Eco*RI site in pNEB193, generating pNEBAd24Orf6. In order to delete the E4 Orf6 gene in pNEBAd24Orf6 and replace it with Ad5 Orf6, pNEBAd24Orf6 was digested with *Sty*I and treated with Klenow to blunt the ends and then digested with to *Eag*I. The desired pNEBAd24Orf6 fragment was then ligated with a PCR product representing the Ad5 Orf6 gene from Ad5 bp 33193 to bp 24125, generating pNEBAd24ΔOrf6Ad5Orf6. The PCR primers used to generate the Ad5 Orf6 fragment (5'CGAGACGGCCGACGCAGATCTGTTTG (SEQ ID NO: 14); 5'GAAGTCCCGGGCTACATGGGGGTAG (SEQ ID NO: 15)) were designed to contain *Eag*I and *Sma*I sites (underlined above) for ligation with the pNEBAd24Orf6 fragment. In the final step pNEBAd24ΔOrf6Ad5Orf6 was digested with *Eco*RI, the restriction fragments were size fractionated by agarose gel electrophoresis and the desired fragment containing Ad5Orf6 flanked by Ad24 sequences was gel purified. Cotransformation of BJ 5183 bacteria with the *Eco*RI fragment and pAd24ΔOrf6BstZ17I, which had been linearized in the E4 region by digestion with *Bst*Z17I, resulted in the generation of pAd24ΔE1ΔOrf6Ad5Orf6 by homologous recombination. Potential clones were screened by restriction analysis and one clone was selected as pre-adenovirus plasmid pAd24ΔE1ΔOrf6Ad5Orf6.

Example 4

Rescue of pAd24ΔE1ΔE4Ad5Orf6, pAd24ΔE1ΔOrf6Ad5Orf6, into Virus

In order to determine if pre-adenovirus plasmids pAd24ΔE1ΔE4Ad5Orf6, pAd24ΔE1ΔOrf6Ad5Orf6, could be rescued into virus and propagated in a group C E1 complementing cell line, the plasmids were each digested with *Pme* I and transfected into T-25 flasks of PER.C6 cells using the calcium phosphate co-precipitation technique; (Cell Phect Transfection Kit, Amersham Pharmacia Biotech Inc.). *Pme*I digestion releases the viral genome from plasmid sequences allowing viral replication to occur after cell entry. Viral cytopathic effect (CPE), indicating that virus replication and amplification was occurring, was observed for both constructs. When CPE was complete, approximately 7-10 days post transfection, the infected cells and media were harvested, freeze/thawed three times and the cell debris pelleted by centrifugation. Approximately 1 ml of the cell lysate was used to infect T-225 flasks of PER.C6 cells at 80-90% confluence. Once CPE was reached, infected cells and media were harvested, freeze/thawed three times and the cell debris pelleted by centrifugation. Clarified cell lysates were then used to infect 2-layer NUNC cell factories of PER.C6 cells. Following complete CPE the virus was purified by ultracentrifugation on CsCl density gradients. In order to verify the genetic structure of the rescued viruses, viral DNA was extracted using pronase treatment followed by phenol chloroform extraction and ethanol precipitation. Viral DNA was then digested with *Hin*dIII and treated with Klenow fragment to end-label the restriction fragments with P33-dATP. The end-labeled restriction fragments were then size-fractionated by gel electrophoresis and visualized by autoradiography. The digestion products were compared with the digestion products of the corresponding pre-Adenovirus plasmid (that had been digested with *Pme*1/*Hind*III prior to labeling) from which they were derived. The expected sizes were observed, indicating that the viruses had been successfully rescued.

Example 5

Comparison of the growth kinetics Ad24 based vectors.

In order to compare the growth kinetic of Ad24ΔE1, Ad24ΔE1Ad5Orf6, Ad24ΔE1ΔE4Ad5Orf6 and Ad24ΔE1ΔOrf6Ad5Orf6 one step growth curves were preformed (Figure 4). PER.C6 cells in 60 mm dishes were infected at 1 vp per cell with either Ad24ΔE1, Ad24ΔE1Ad5Orf6, Ad24ΔE1ΔE4Ad5Orf6 or Ad24ΔE1ΔOrf6Ad5Orf6. Cells and media were then harvested at various times post infection, freeze thawed three times and clarified by centrifugation. The amount of virus present in the samples was determined by quantitative PCR and is illustrated in Figure 4. This study demonstrates that Ad24 vectors that incorporate Ad5

Orf6 have a distinct growth advantage over Ad24ΔE1 in PER.C6 cells. The instant invention can be practiced with recombinant Ad24 vectors absent a heterologous Orf 6 region where the E1-complementing cell line expresses an Ad24 E1 region or, alternatively, E1 and E4 regions of the same serotype (such as Ad5E1/E4-expressing cell lines).

Example 6

Insertion of an Expression Cassette into pAd24ΔE1ΔE4Ad5Orf6, pAd24ΔE1ΔOrf6Ad5Orf6,

In order to introduce a gag or SEAP expression cassette (*see* Figures 13 and 14, respectively) into the E1 region of the Ad24 pre-Adenovirus plasmids described above (pAd24ΔE1ΔE4Ad5Orf6, pAd24ΔE1ΔOrf6Ad5Orf6) bacterial recombination was used. A gag expression cassette consisting of the following: 1) the immediate early gene promoter from the human cytomegalovirus, 2) the coding sequence of the human immunodeficiency virus type 1 (HIV-1) gag (strain CAM-1; 1526 bp) gene, and 3) the bovine growth hormone polyadenylation signal sequence, was cloned into the E1 deletion in Ad17 shuttle plasmid, pABSAd17-3, generating pABSAd17HCMVgagBGHpA. The ITR cassette contains sequences from the right (bp 34469 to 35098) and left (bp 4 to 414 and bp 3373 to 4580) end of the Ad17 genome separated by plasmid sequences containing a bacterial origin of replication and an Ampicillin resistance gene. The ITR cassette contains a deletion of E1 sequences from Ad17 (bp 415 to 3372) with a unique *Swa* I site located in the deletion. The gag expression cassette was obtained from a previously constructed shuttle plasmid by EcoRI digestion. Following the digestion the desired fragment was gel purified, treated with Klenow to obtain blunt ends and cloned into the SwaI site in pABSAd17-3. This cloning step resulted in the gag expression cassette being cloned into the E1 deletion between bp 414 and 3373 in the E1 parallel orientation. The shuttle vector containing the gag transgene was digested to generate a DNA fragment consisting of the gag expression cassette flanked by Ad17 bp 4 to 414 and bp 3373 to 4580 and the fragment was purified after electrophoresis on an agarose gel. Cotransformation of BJ 5183 bacteria with the shuttle vector fragment and one of the Ad24 pre-Ad plasmids (pAd24ΔE1ΔE4Ad5Orf6, pAd24ΔE1ΔOrf6Ad5Orf6,), linearized in the E1 region by digestion with *Swa* I, resulted in the generation of the corresponding Ad24 gag-containing pre-Adenovirus plasmids (pAd24ΔE1gagΔE4Ad5Orf6, pAd24ΔE1gagΔOrf6Ad5Orf6) by homologous recombination. Potential clones were screened by restriction analysis.

A similar strategy was used to generate Ad24 pre-Ad plasmids containing a SEAP expression cassette. In this case a SEAP expression cassette consisting of: 1) the immediate early gene promoter from the human cytomegalovirus, 2) the coding sequence of the human

placental SEAP gene, and 3) the bovine growth hormone polyadenylation signal sequence was cloned into the E1 deletion in Ad17 shuttle plasmid, pABSAd17-3, generating pABSAd17HCMVSEAPBGH. The SEAP expression cassette was obtained from a previously constructed shuttle plasmid by EcoRI digestion. Following the digestion the desired fragment was gel purified, treated with Klenow to obtain blunt ends and cloned into the *Swa*I site in pABSAd17-3. The shuttle vector containing the SEAP transgene was digested to generate a DNA fragment consisting of the SEAP expression cassette flanked by Ad17 bp 4 to 414 and bp 3373 to 4580 and the fragment was purified after electrophoresis on an agarose gel. Cotransformation of BJ 5183 bacteria with the shuttle vector fragment and one of the Ad24 pre-Ad plasmids (pAd24ΔE1ΔE4Ad5Orf6, pAd24ΔE1ΔOrf6Ad5Orf6,), linearized in the E1 region by digestion with *Swa* I, resulted in the generation of the corresponding Ad24 SEAP-containing pre-Adenovirus plasmids (pAd24ΔE1SEAPΔE4Ad5Orf6, pAd24ΔE1SEAPΔOrf6Ad5Orf6) by homologous recombination. Potential clones were screened by restriction analysis. All pre-Ad plasmids were rescued into virus and expanded to prepare CsCl purified stocks as described above.

Example 7

In Vivo Immunogenicity

A. Immunization

Cohorts of 3-6 animals were given intramuscular injections at wk 0 and wk 4 of either of the following constructs: (1) 10^11 vp MRKAd5-HIV1 gag; (2) 10^10 vp MRKAd5-HIV1 gag; (3) 10^11 vp of Ad24ΔE1gagΔOrf6Ad5Orf6; (4) 10^10 vp of Ad24ΔE1gagΔOrf6Ad5Orf6; or (5) 10^10 vp of Ad24ΔE1gagΔE4Ad5Orf6. Rhesus macaques were between 3-10 kg in weight. In all cases, the total dose of each vaccine was suspended in 1 mL of buffer. The macaques were anesthetized (ketamine/xylazine) and the vaccines were delivered i.m. in 0.5-mL aliquots into both deltoid muscles using tuberculin syringes (Becton-Dickinson, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMC) were prepared from blood samples collected at several time points (typically 4 wk intervals) during the immunization regimen. All animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council.

B. ELISPOT Assay

The IFN-γ ELISPOT assays for rhesus macaques were conducted following a previously described protocol (Allen et al., 2001 *J. Virol.* 75(2):738-749; Casimiro et al., 2002 *J. Virol.* 76:185-94), with some modifications. For antigen-specific stimulation, a peptide pool was prepared from 20-aa peptides that encompass the entire HIV-1 gag sequence with 10-aa overlaps (Synpep Corp., Dublin, CA). To each well, 50 μL of 2-4 x 10^5 peripheral blood mononuclear cells (PBMCs) were added; the cells were counted using Beckman Coulter Z2 particle analyzer with a lower size cut-off set at 80 femtoliters ("fL"). Either 50 μL of media or the gag peptide pool at 8 μg/mL concentration per peptide were added to the PBMC. The samples were incubated at 37°C, 5% CO_2 for 20-24 hrs. Spots were developed accordingly and the plates were processed using custom-built imager and automatic counting subroutine based on the ImagePro platform (Silver Spring, MD); the counts were normalized to 10^6 cell input.

C. Intracellular Cytokine Staining.

To 1 ml of 2 x 10^6 PBMC/mL in complete RPMI media (in 17x100mm round bottom polypropylene tubes (Sarstedt, Newton, NC)), anti-hCD28 (clone L293, Becton-Dickinson) and anti-hCD49d (clone L25, Becton-Dickinson) monoclonal antibodies were added to a final concentration of 1 μg/mL. For gag-specific stimulation, 10 μL of the peptide pool (at 0.4 mg/mL per peptide) were added. The tubes were incubated at 37 °C for 1 hr., after which 20 μL of 5 mg/mL of brefeldin A (Sigma) were added. The cells were incubated for 16 hr at 37 °C, 5% CO_2, 90% humidity. 4 mL cold PBS/2%FBS were added to each tube and the cells were pelleted for 10 min at 1200 rpm. The cells were re-suspended in PBS/2%FBS and stained (30 min, 4 °C) for surface markers using several fluorescent-tagged mAbs: 20 μL per tube anti-hCD3-APC, clone FN-18 (Biosource); 20 μL anti-hCD8-PerCP, clone SK1 (Becton Dickinson); and 20 μL anti-hCD4-PE, clone SK3 (Becton Dickinson). Sample handling from this stage was conducted in the dark. The cells were washed and incubated in 750 μL 1xFACS Perm buffer (Becton Dickinson) for 10 min at room temperature. The cells were pelleted and re-suspended in PBS/2%FBS and 0.1 μg of FITC-anti-hIFN-γ, clone MD-1 (Biosource) was added. After 30 min incubation, the cells were washed and re-suspended in PBS. Samples were analyzed using all four color channels of the Becton Dickinson FACSCalibur instrument. To analyze the data, the low side- and forward-scatter lymphocyte population was initially gated; a common fluorescence cut-off for cytokine-positive events was used for both $CD4^+$ and $CD8^+$ populations, and for both mock and gag-peptide reaction tubes of a sample.

D. Anti-p24 ELISA.

A modified competitive anti-p24 assay was developed using reagents from the Coulter p24 Antigen Assay kit (Beckman Coulter, Fullerton, CA). Briefly, to a 250-μL serum sample, 20 μL of Lyse Buffer and 15 μL of p24 antigen (9.375 pg) from the Coulter kit were added. After mixing, 200 μL of each sample were added to wells coated with a mouse anti-p24 mAb from the Coulter kit and incubated for 1.5 hr at 37°C. The wells were then washed and 200 μL of Biotin Reagent (polyclonal anti-p24-biotin) from the Coulter kit was added to each well. After a 1 hr, 37°C incubation, detection was achieved using strepavidin-conjugated horseradish peroxidase and TMB substrate as described in the Coulter Kit. OD_{450nm} values were recorded. A 7-point standard curve was generated using a serial 2-fold dilution of serum from an HIV-seropositive individual. The lower cut-off for the assay is arbitrarily set at 10 milli Merck units/mL (mMU/mL) defined by a dilution of the seropositive human serum. This cutoff falls at approximately 65% of the maximum bound control signal which corresponds to that obtained with the diluent control only and with no positive analyte.

E. Results

PBMCs collected at regular 4-wk intervals were analyzed in an ELISPOT assay (Figure 6). Both Ad24ΔE1gagΔOrf6Ad5Orf6 and Ad24ΔE1gagΔE4Ad5Orf6 were able to induce significant levels of gag-specific T cells in non-human primates. At 10^11 vp dose level, the Ad24-induced responses were within 2-3-fold of those of MRKAd5-HIV1 gag. Both Ad24 vectors were also able to induce detectable levels of gag-specific T cells at 10^10 vp but were lower than those observed using MRKad5gag at the same dose.

PBMCs collected at wk 12 from the vaccinees were analyzed for intracellular IFN-γ staining after the priming immunizations. The assay results provided information on the relative amounts of $CD4^+$ and $CD8^+$ gag-specific T cells in the peripheral blood (Figure 7). The results indicated that the prime-boost immunization approach was able to elicit in rhesus macaques both HIV-specific $CD4^+$ and $CD8^+$ T cells.

F. Humoral Immune Responses.

The Ad24-based vaccine vector was able to generate detectable levels of circulating anti-gag antibodies at the reasonably high dose level (Figure 8). No detectable titers were observed at equal to or lower than 10^10 vp, suggesting the existence of a dose-dependent response.

Example 8

In Vivo Transgene Expression

A. Immunization

Cohorts of 5 C3H/HeN mice were given single intramuscular injections of one of the following vectors: (1) 10^10 vp Ad24ΔE1SEAPΔE4Ad5Orf6; (2) 10^10 vp Ad24ΔE1SEAPΔOrf6Ad5Orf6; (3) 10^10 vp MRKAd5SEAP; and (4) 10^9 vp MRKAd5SEAP. Female mice were between 4-10 weeks old. The total dose of each vaccine was suspended in 0.1 mL of buffer. The vectors were given to both quadriceps of each of the animals with a volume of 50 uL per quad and using 0.3-mL 28G1/2 insulin syringes (Becton-Dickinson, Franklin Lakes, NJ). For the primates, the total dose of each vaccine was suspended in 1 mL of buffer. The monkeys were anesthetized (ketamine/xylazine mixture) and the vaccines were delivered i.m. in 0.5-mL aliquots into two muscle sites using tuberculin syringes (Becton-Dickinson, Franklin Lakes, NJ). Serum samples were collected at defined intervals and stored frozen until the assay date. All animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council.

B. SEAP Assay

Serum samples were analyzed for circulating SEAP levels using TROPIX phospha-light chemiluminescent kit (Applied Biosystems Inc). Duplicate 5 uL aliquots of each serum were mixed with 45 uL of kit-supplied dilution buffer in a 96-well white DYNEX plate. Serially diluted solutions of a human placental alkaline phosphatase (Catalog no. M5905, Sigma, St. Louis, MO) in 10% naïve monkey serum served to provide the standard curve. Endogenous SEAP activity in the samples was inactivated by heating the wells for 30 minutes at 65 °C. Enzymatic SEAP activities in the samples were determined following the procedures described in the kit. Chemiluminescence readings (in relative light units) were recorder using DYNEX luminometer. RLU readings are converted to ng/mL SEAP using a log-log regression analyses.

C. Rodent Results

Serum samples prior to and after the injection were analyzed for circulating SEAP activities and the results are shown in Figure 9. Results indicate that (1) both Ad24 constructs are all capable of expressing the SEAP transgene *in vivo* to comparable levels; and that (2) the level of expression achieved using the Ad24 vectors are comparable to that of Ad5 at 10-fold

lower dose. The levels of SEAP in the serum dropped dramatically after day 2 and were at background levels by day 12.

D. Primate Results

Cohorts of 3 rhesus macaques were given single intramuscular injections of one of the following vectors: (1) 10^11 vp MRKAd5-SEAP; (2) 10^9 vp MRKAd5-SEAP; (3) 10^11 vp Ad24ΔE1SEAPΔOrf6Ad5Orf6; or (4) 10^11 vp Ad24ΔE1SEAPΔE4Ad5Orf6. Serum samples prior to and after the injection were analyzed for circulating SEAP activities and the results are shown in Figure 10.

Results indicate that the peak levels of SEAP product produced by adenovirus serotype 24 were lower than but were within 3-fold of that of MRKAd5 at the same high dose level of 10^11 vp (Figure 10). The levels observed with adenovirus serotype 24 are generally 50-fold higher than those observed using 10^9 vp of MRKAd5. The levels of SEAP in the serum dropped dramatically after day 10 and were close to background as early as day 15. These observations strongly indicate that adenovirus serotype 24 is very efficient in expressing a transgene following intramuscular administration in a primate.

Example 9

Construction of pMRKAd24ΔE1ΔE4Ad5Orf6

To construct pMRKAd24ΔE1ΔE4Ad5Orf6 (An Ad24 pre-Ad plasmid, composed entirely of Ad24 sequence and containing an E1 deletion and an E4 deletion substituted with Ad5 Orf6), an Ad24 ITR cassette was constructed containing sequences from the right (bp 31978 to 32264 and bp 34713 to 35164) and left (bp 4 to 450 and bp 3364 to 3799) end of the Ad24 genome separated by plasmid sequences containing a bacterial origin of replication and an ampicillin resistance gene. These four segments were generated by PCR and cloned sequentially into pNEB193, generating pNEBAd24-4. Next the Ad5 Orf6 open reading frame (Ad5 bp 31192 to bp 34078) was generated by PCR and cloned between Ad24 bp 32264 and 34713 generating pNEBAd24E-Ad5Orf6 (the ITR cassette). PNEB193 is a commonly used commercially available cloning plasmid (New England Biolabs cat# N3051S) containing a bacterial origin of replication, ampicillin resistance gene and a multiple cloning site into which the PCR products were introduced. The ITR cassette contains a deletion of E1 sequences from Ad24 bp 451 to 3363 with a unique Swa I restriction site located in the deletion and an E4 deletion from Ad24 bp 32265 to 34712 into which Ad5 Orf6 was introduced in an E4 parallel orientation. In this construct Ad5 Orf6 expression is driven by the Ad24 E4 promoter. The Ad24 sequences (bp 31978 to 32264 and bp 3464 to 3799) in the ITR cassette provide regions of homology with the

purified Ad24 viral DNA in which bacterial recombination can occur following cotransformation into BJ 5183 bacteria (Figure 11). The ITR cassette was also designed to contain unique restriction enzyme sites (PmeI) located at the end of the viral ITR's so that digestion will release the recombinant Ad24 genome from plasmid sequences. Potential clones will be screened by restriction analysis and one clone was selected as pMRKAd24ΔE1ΔE4Ad5Orf6. Pre-Adenovirus plasmid pMRKAd24ΔE1ΔE4Ad5Orf6 should contain Ad24 sequences from bp 4 to 450; bp 3364 to bp 32264 and bp 34713 to bp 35164 with Ad5Orf6 cloned between bp 32264 and bp 34713. The bp numbering in the above description refers to the wt sequence for both Ad24 and Ad5.

Example 10

Insertion of HIV-1 gag and SEAP transgenes into pAd24ΔE1ΔE4Ad5Orf6

In order to introduce a gag or SEAP expression cassettes into the E1 region of pMRKAd24ΔE1ΔE4Ad5Orf6, bacterial recombination will be used. An HIV-1 gag expression cassette will consist of the following: 1) the immediate early gene promoter from the human cytomegalovirus, 2) the coding sequence of the human immunodeficiency virus type 1 (HIV-1) gag (strain CAM-1; 1526 bp) gene, and 3) the bovine growth hormone polyadenylation signal sequence, in the E1 deletion of an Ad24 shuttle plasmid, pNEBAd24-2 (a precursor to the Ad24 ITR cassette described above), generating pNEBAd24CMVgagBGHpA. PNEBAd24-2 contains Ad24 sequences from the left end of the genome (bp 4 to 450 and bp 3364 to 3799) that define the E1 deletion. The gag expression cassette will be obtained from a previously constructed plasmid and cloned into the E1 deletion between bp 450 and 3364 in the E1 parallel orientation. The shuttle vector containing the gag transgene will be digested to generate a DNA fragment consisting of the gag expression cassette flanked by Ad24 bp 4 to 450 and bp 3364 to 3799 and the fragment will be purified after electrophoresis on an agarose gel. Cotransformation of BJ 5183 bacteria with the shuttle vector fragment and pMRKAd24ΔE1ΔE4Ad5Orf6 which was linearized in the E1 region by digestion with SwaI, should result in the generation of Ad24 gag-containing pre-Adenovirus plasmids pMRKAd24ΔE1gagΔE4Ad5Orf6 by homologous recombination. Potential clones will be screened by restriction analysis.

A similar strategy will be used to generate Ad24 pre-Ad plasmids containing a SEAP expression cassette. In this case, a SEAP expression cassette will consist of: 1) the immediate early gene promoter from the human cytomegalovirus, 2) the coding sequence of the human placental SEAP gene, and 3) the bovine growth hormone polyadenylation signal sequence cloned into the E1 deletion of an Ad24 shuttle plasmid, pNEBAd24-2, generating

pNEBAd24CMVSEAPBGHpA. The transgene will then be recombined into pMRKAd24ΔE1ΔE4Ad5Orf6 as described above for the gag transgene.

Example 11

In Vivo *Immunogenicity*

A. Immunization

Rhesus macaques were between 3-10 kg in weight. In all cases, the total dose of each vaccine was suspended in 1 mL of buffer. The macaques were anesthetized (ketamine/xylazine) and the vaccines were delivered i.m. in 0.5-mL aliquots into both deltoid muscles using tuberculin syringes (Becton-Dickinson, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMC) were prepared from blood samples collected at several time points during the immunization regimen. All animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council.

B. T Cell Responses

Ad24 Vaccine Vector as a Heterologous Booster: Cohort of 4 rhesus macaques was immunized initially with 3 doses (wk 0, 4, 26) of either 10^7 or 10^9 vp of MRKAd5-gag (*see* Figures 16A-16AX) or MRKAd6-gag(*see* Figures 17A-17N). At wk 56, the animals received a booster vaccine of 10^{11} vp Ad24ΔE1gagΔOrf6Ad5Orf6. A separate cohort of naïve animals received a single dose of the booster vaccine. The results of the IFN-γ ELISPOT analyses of PBMC collected during the course of the studies are shown in Figure 18. It is apparent that the Ad24 HIV vectors can be utilized to amplify the existing pools of HIV-specific T cells. The increases in the levels of gag-specific T cells from the pre-boost levels to those measured at 4 wks post boost were consistently larger than the levels induced by the same booster vaccine in naïve animals. PBMCs from the vaccinees of the heterologous MRKAd5/MRKAd6-Ad24 boost regimen were analyzed for intracellular IFN-γ staining after the priming immunizations (wk 60). The assay results provided information on the relative amounts of $CD4^+$ and $CD8^+$ gag-specific T cells in the peripheral blood (Figure 19). The results indicated that heterologous prime-boost immunization approach was able to elicit in rhesus macaques both HIV-specific CD4+ and CD8+ T cells.

Ad24 Vaccine Vector as a Heterologous Primer: In a separate study, a cohort of 3 rhesus macaques was immunized initially with 2 doses (wk 0, 4) of 10^{11} vp Ad24ΔE1gagΔOrf6Ad5Orf6 and boosted at wk 24 with 10^7 vp of MRKAd5-gag. The low dose

of MRKAd5-gag is selected to mimic the effect of pre-existing neutralizing immunity to the vector in a subject. A separate cohort of naïve animals was given a single dose of 10^7 vp MRKAd5-gag. The results of the IFN-γ ELISPOT analyses of PBMC collected during the course of the studies are shown in Figure 20.

The Ad24-based vaccine was able to prime effectively for HIV-specific T cell responses in macaques. Boosting with a low dose MRKAd5-gag resulted in a significant increase in the levels of gag-specific T cells. The increases in 2 out of 3 animals exceed the levels typically observed after treatment of naïve animals with the same low dose of MRKAd5-gag.